DNA MOLECULES AND PROTEIN DISPLAYING IMPROVED TRIAZINE COMPOUND DEGRADING ABILITY

Background of the Invention

More than 8 million organic compounds are known and many are thought to be biodegradable by microorganisms, the principle agents for recycling organic matter on Earth. In this context, microbial enzymes represent the greatest diversity of novel catalysts. This is why microbial enzymes are predominant in industrial enzyme technology and in bioremediation, whether used as purified enzymes or in whole cell systems.

There is increased interest in engineering bacterial enzymes for improved industrial performance. For example, site directed mutagenesis of subtilisin has resulted in the development of enzyme variants with improved properties for use in detergents. Most applied enzymes, particularly those used in biodegrading pollutants, however, are naturally evolved. That is, they are unmodified from the form in which they were originally present in a soil bacterium.

For example, most bioremediation is directed against petroleum hydrocarbons, pollutants that are natural products and thus have provided selective pressure for bacterial enzyme evolution over millions of years. Synthetic compounds not resembling natural products are more likely to resist biodegradation and hence accumulate in the environment. This changes over a bacterial evolutionary time scale; compounds considered to be non-biodegradable several decades ago, for example PCBs and tetrachloroethylene, are now known to biodegrade. This is attributed to recent evolution and dispersal of the newly evolved gene(s) throughout microbial populations by mechanisms such as conjugative plasmids and transposable DNA elements.

A better understanding of the evolution of new biodegradative enzymes will reveal how nature cleanses the biosphere. Furthermore, the ability to emulate the process in the laboratory may shave years off the lag period between the introduction of a new molecular compound into the environment and the development of a dispersed microbial antidote that will remove it.

Atrazine [2-chloro-4-(ethylamino)-6-(isopropylamino)-1,3,5-triazine)] is a widely used *s*-triazine (i.e., symmetric triazine) herbicide for the control of broad-leaf weeds. Approximately 800 million pounds were used in the United States between 1980 and 1990. As a result of this widespread use, for both selective and nonselective weed control, atrazine and other *s*-triazine-containing compounds have been detected in ground and surface water in several countries.

Numerous studies on the environmental fate of atrazine have shown that atrazine is a recalcitrant compound that is transformed to CO_2 very slowly, if at all, under aerobic or anaerobic conditions. It has a water solubility of 33 mg/l at 27°C. Its half-life (i.e., time required for half of the original concentration to dissipate) can vary from about 4 weeks to about 57 weeks when present at a low concentration (i.e., less than about 2 parts per million (ppm)) in soil. High concentrations of atrazine, such as those occurring in spill sites have been reported to dissipate even more slowly.

As a result of its widespread use, atrazine is often detected in ground water and soils in concentrations exceeding the maximum contaminant level (MCL) of 3 μg/l (i.e., 3 parts per billion (ppb)), a regulatory level that took effect in 1992. Point source spills of atrazine have resulted in levels as high as 25 ppb in some wells. Levels of up to 40,000 mg/l (i.e., 40,000 parts per million (ppm)) atrazine have been found in the soil at spill sites more than ten years after the spill incident. Such point source spills and subsequent runoff can cause crop damage and ground water contamination.

There have been numerous reports on the isolation of *s*-triazine-degrading microorganisms (see, e.g., Behki et al., J. Agric. Food Chem., 34, 746-749 (1986); Behki et al., Appl. Environ. Microbiol., 59, 1955-1959 (1993); Cook, FEMS Microbiol. Rev., 46, 93-116 (1987); Cook et al., J. Agric. Food Chem., 29, 1135-1143 (1981); Erickson et al., Critical Rev. Environ. Cont., 19, 1-13 (1989); Giardina et al., Agric. Biol. Chem., 44, 2067-2072 (1980); Jessee et al., Appl. Environ. Microbiol., 45, 97-102 (1983); Mandelbaum et al., Appl. Environ. Microbiol., 61, 1451-1457 (1995); Mandelbaum et al., Appl. Environ. Microbiol., 59, 1695-1701 (1993); Mandelbaum et al., Environ. Sci. Technol.,

27, 1943-1946 (1993); Radosevich et al., Appl. Environ. Microbiol., 61, 297-302 (1995); and Yanze-Kontchou et al., Appl. Environ. Microbiol., 60, 4297-4302 (1994)). Many of the organisms described, however, failed to mineralize atrazine (see, e.g., Cook, FEMS Microbiol. Rev., 46, 93-116 (1987); and Cook et al., J. Agric. Food Chem., 29, 1135-1143 (1981)). While earlier studies have reported atrazine degradation only by mixed microbial consortia, more recent reports have indicated that several isolated bacterial strains can degrade atrazine. In fact, research groups have identified atrazine-degrading bacteria classified in different genera from several different locations in the U.S. (e.g., Minnesota, Iowa, Louisiana, and Ohio) and Switzerland (Basel).

An atrazine-degrading bacterial culture, identified as *Pseudomonas* sp. strain ADP (Mandelbaum et al., Appl. Environ. Microbiol., 61, 1451-1457 (1995); Mandelbaum et al., Appl. Environ. Microbiol., 59, 1695-1701 (1993); de Souza et al., J. Bact., 178, 4894-4900 (1996); and Mandelbaum et al., Environ. Sci. Technol., 27, 1943-1946 (1993)), was isolated and was found to degrade atrazine at concentrations greater than about 1,000 µg/ml under growth and non-growth conditions. See also, Radosevich et al., Appl. Environ. Microbiol., 61, 297-302 (1995) and Yanze-Kontchou et al., Appl. Environ. Microbiol., 60, 4297-4302 (1994). *Pseudomonas* sp. strain ADP (Atrazine Degrading *Pseudomonas*) uses atrazine as a sole source of nitrogen for growth. The organism completely mineralizes the *s*-triazine ring of atrazine under aerobic growth conditions. That is, this bacteria is capable of degrading the *s*-triazine ring and mineralizing organic intermediates to inorganic compounds and ions (e.g., CO_2).

The genes that encode the enzymes for MELAMINE (2,4,6-triamino-*s*-triazine) metabolism have been isolated from a *Pseudomonas* sp. strain. The genes that encode atrazine degradation activity have been isolated from *Rhodococcus* sp. strains; however, the reaction results in the dealkylation of atrazine. In addition, the gene that encodes atrazine dechlorination has been isolated from a *Pseudomonas* sp. strain. See, for example, de Souza et al., Appl. Environ. Microbiol., 61, 3373 (1995). The protein expressed by the gene disclosed by de Souza et al., degrades atrazine, for example, at a V_{max} of about 2.6 µmol of hydroxyatrazine per min per mg protein. Although this is

significant, it is desirable to obtain genes and the proteins they express that are able to dechlorinate triazine-containing compounds with chlorine moieties at an even higher rate and/or under a variety of conditions, such as, but not limited to, conditions of high temperature (e.g., at least about 45°C and preferably at least about 65°C), various pH conditions, and/or under conditions of high salt content (e.g., about 20-30 g/L), or under other conditions in which the wild type enzyme is not stable, efficient, or active. Similarly, it is desirable to obtain genes and proteins encoded by these genes that degrade triazine-containing compounds such as those triazine containing compounds available under the trade names; "AMETRYN", "PROMETRYN", "CYANAZINE", "MELAMINE", "SIMAZINE", as well as TERBUTHYLAZINE and desethyldesisopiopylatriazine. It is also desirable to identify proteins expressed in organisms that degrade triazine-containing compounds in the presence of other nitrogen sources such as ammonia and nitrate.

Summary of the Invention

The present invention provides isolated and purified DNA molecules that encode atrazine degrading enzymes similar to, but having different catalytic activities from a wild type (i.e., from an isolated but naturally occurring atrazine chlorohydrolase). The term "altered enzymatic activities" is used to refer to homologs of atrazine chlorohydrolase having altered catalytic rates as quantitated by k_{cat} and K_m, improved ability to degrade atrazine, altered substrate ranges, altered activities as compared to the native sequence in aqueous solutions, altered stability in solvents, altered active temperature ranges or altered reaction conditions such as salt concentration, pH, improved activity in a soil environment, and the like, as compared with the wild-type atrazine chlorohydrolase (AtzA) protein.

In one preferred embodiment, the present invention provides DNA fragments encoding a homolog of atrazine chlorhydrolase and comprising the sequence selected from the group consisting of SEQ ID NO:3, SEQ ID NO:4, SEQ ID NOS:7-11 and SEQ ID NOS: 17-21. In one embodiment the invention relates to these DNA fragments in a vector, preferably an expression vector.

Further, the invention relates to the DNA fragment in a cell. In one embodiment the cell is a bacterium and in a preferred embodiment, the bacterium is *E. coli.*

The invention also relates to *s*-triazine-degrading proteins having at least one amino acid different from the protein of SEQ ID NO:2, wherein the coding region of the nucleic acid encoding the *s*-triazine degrading protein has at least 95% homology to SEQ ID NO:1 and wherein the *s*-triazine-degrading protein has an altered catalytic activity as compared with the protein having the sequence of SEQ ID NO:2. In one embodiment, the protein is selected from the group consisting of SEQ ID NOS: 5, 6 and 22-26. In one embodiment the substrate for the *s*-triazine degrading protein is ATRAZINE. In another embodiment the substrate for the *s*-triazine degrading protein is TERBUTHYLAZINE and in yet another embodiment the substrate for the *s*-triazine degrading protein is MELAMINE. In another embodiment this invention relates to a remediation composition comprising a cell producing at least one *s*-triazine-degrading protein having at least one amino acid different from the protein of SEQ ID NO:2, wherein the coding region of the nucleic acid encoding the *s*-triazine degrading protein has at least 95% homology to SEQ ID NO:1 and wherein the *s*-triazine-degrading protein has an altered catalytic activity as compared with the protein having the sequence of SEQ ID NO:2. In a preferred embodiment the composition is suitable for treating soil or water. In another embodiment the remediation composition comprises at least one s-triazine-degrading protein having at least one amino acid different from the protein of SEQ ID NO:2, wherein the coding region of the nucleic acid encoding the *s*-triazine degrading protein has at least 95% homology to SEQ ID NO:1 and wherein the *s*-triazine-degrading protein has an altered catalytic activity as compared with the protein having the sequence of SEQ ID NO:2. In a preferred embodiment this composition is also suitable for treating soil or water. In one embodiment the remediation composition comprises the protein bound to an immobilization support. In yet another embodiment, these proteins are homotetramers, such as the homotetramers formed by AtzA.

In another embodiment the invention relates to a protein selected from the group consisting of proteins comprising the amino acid sequences of SEQ ID NOS: 5, 6 and 22-26.

Sub B1 In another aspect of this invention, the invention relates to a DNA fragment having a portion of its nucleic acid sequence having at least 95% homology to a nucleic acid sequence consisting of position 236 and ending at position 1655 of SEQ ID NO:1, wherein the DNA fragment is capable of hybridizing under stringent conditions to SEQ ID NO:1 and wherein there is at least one amino acid change in the protein encoded by the DNA fragment as compared with SEQ ID NO:2 and wherein the protein encoded by the DNA fragment is capable of dechlorinating at least one s-triazine-containing compound and has a catalytic activity different from the enzymatic activity of the protein of SEQ ID NO:2. In one embodiment the *s*-triazine-containing compound is ATRAZINE, TERBUTHYLAZINE, or MELAMINE. In one embodiment.

Sub B2 The invention also relates to a method for treating a sample comprising an *s*-triazine containing compound comprising the step of adding a adding a protein to a sample comprising an *s*-triazine-containing compound wherein the protein is encoded by gene having at least a portion of the nucleic acid sequence of the gene having at least 95% homology to the sequence beginning at position 236 and ending at position 1655 of SEQ ID NO:1, wherein the gene is capable of hybridizing under stringent conditions to SEQ ID NO:1, wherein there is at least one amino acid change in the protein encoded by the DNA fragment as compared with SEQ ID NO:2 and wherein the protein has an altered catalytic activity as compared to the protein having the amino acid sequence of SEQ ID NO:2. In one embodiment, the composition comprises bacteria expressing the protein. In one embodiment the *s*-triazine -containing compound is atrazine, in another the *s*-triazine-containing compound is TERBUTHYLAZINE and in another the *s*-triazine containing compound is (2,4,6-triamino-*s*-triazine). In one embodiment, the protein encoded by the gene is selected from the group consisting of SEQ ID NOS: 5, 6 and 22-26.

In another aspect, this invention relates to a method for obtaining homologs of an atrazine chlorohydrolase comprising the steps of obtaining a nucleic acid sequence encoding atrazine chlorohydrolase, mutagenizing the nucleic acid to obtain a modified nucleic acid sequence that encodes for a protein having an amino acid sequence with at least one amino acid change relative to the amino acid sequence of the atrazine chlorohydrolase, screening the proteins encoded by the modified nucleic acid sequence; and selecting proteins with altered catalytic activity as compared to the catalytic activity of the atrazine chlorohydrolase. Preferably, the atrazine chlorohydrolase nucleic acid sequence is SEQ ID NO:1. In one embodiment the altered catalytic activity is an improved ability to degrade ATRAZINE. In another embodiment, the altered catalytic activity is an altered substrate activity.

Other homologs with an improved rate of catalytic activity for atrazine include clones A40, A42, A44, A46 and A60 having nucleic acid sequences (SEQ ID NOS:17-21, respectively). Other homologs capable of better degrading TERBUTHYLAZINE include A42, A44, A46 and A60 as well as A11 and A13.

Brief Description of the Drawings

Fig. 1. Nucleotide sequence alignment of wild type *atzA* (bottom sequence) from *Pseudomonas sp.* strain ADP and clone (A7) (SEQ ID NO:1 and SEQ ID NO:3).

Fig. 2. Nucleotide sequence alignment of wild type *atzA* (bottom sequence) from *Pseudomonas sp.* strain ADP and clone (T7) (SEQ ID NO: 1 and SEQ ID NO:4).

Fig. 3. Amino acid sequence alignment of wild type AtzA (bottom sequence) from *Pseudomonas sp.* strain ADP and clone (A7) (SEQ ID NO:2 and SEQ ID NO:5).

Fig. 4. Amino acid sequence alignment of wild type AtzA from *Pseudomonas sp.* strain ADP and clone (T7) (SEQ ID NO:2 and SEQ ID NO:6).

Fig. 5. Nucleotide sequence alignment of wild type *atzA* (SEQ ID NO:1, bottom sequence) from *Pseudomonas sp.* strain ADP and clone (A11). Fig. 5(a) provides the sequence from nucleic acids 11-543 (SEQ ID NO:7), Fig. 5(b) provides the sequence from nucleic acids 454-901 (SEQ ID NO:8), Fig. 5(c) provides the sequence from 1458-1851 (SEQ ID NO:9; N in this sequence indicates that this nucleotide has not been verified) and Fig. 5(d) provides the sequence from nucleic acids 1125-1482 (SEQ ID NO:10) of clone A11. The "N" in these sequences refer to nucleic acids that are being verified.

Fig. 6. Nucleotide sequence alignment of a portion of the nucleic acid sequence of wild type *atzA* from *Pseudomonas sp.* strain ADP and nucleic acids 436-963 of clone (A13) (SEQ ID NO:11 and SEQ ID NO:1).

Fig. 7. is a histogram illustrating the TERBUTHYLAZINE degradative ability of two homologs of this invention (T7= sample 3 and A7 = sample 4). Fig. 7(a) illustrates the % of TERBUTHYLAZINE remaining after exposure to AtzA or a homolog. Fig. 7(b) illustrates the relative amount of hydroxyterbuthylazine as a measure of TERBUTHYLAZINE degradation.

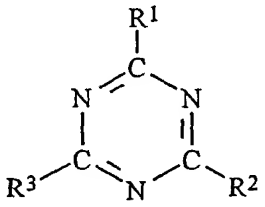
Fig. 8. is another set of histograms illustrating the terbutylazine degradative ability of three homologs A7, All, and T7. Figure 8(a) provides the % of TERBUTHYLAZINE remaining after a 15 minute exposure to the homolog in the presence or absence of the metals and additives of Samples 1-10. Figure 8(b) provides the relative amount of hydroxterbuthylazine in the presence or absence of the metals and compounds of Samples 1-10.

Fig. 9. is a comparison of PCR amplified fragments using two primers of the atrazine hydrochlorase gene from 6 different types of bacteria; *Pseudomonas* sp. strain ADP; *Ralstonia* strain M91-3; *Clavibacter* (*Clav.*); *Agrobacterium* strain J14(a); ND (an organism with no genus assigned) strain 38/38; and *Alcaligenes* strain SG1 (SEQ ID NOS: 12-16).

Detailed Description of the Invention

The present invention provides isolated and purified DNA molecules, and isolated and purified proteins, involved in the degradation of *s*-triazine-containing compounds. The proteins encoded by the genes of this

invention are involved in the dechlorination and/or the deamination of *s*-triazine-containing compounds. The wild type AtzA protein can catalyze the dechlorination of *s*-triazine-containing compounds but not the deamination of these compounds. The dechlorination reaction occurs on *s*-triazine containing compounds that include a chlorine atom and at least one alkylamino side chain. Such compounds have the following general formula:



wherein R^1 = Cl, $R^2 = NR^4R^5$ (wherein R^4 and R^5 are each independently H or a C_{1-3} alkyl group), and $R^3 = NR^6R^7$ (wherein R^6 and R^7 are each independently H or a C_{1-3} alkyl group), with the proviso that at least one of R^2 or R^3 is an alkylamino group. As used herein, an "alkylamino" group refers to an amine side chain with one or two alkyl groups attached to the nitrogen atom. Examples of such compounds include atrazine (2-chloro-4-ethylamino-6-isopropylamino-1,3,5-*s*-triazine), desethylatrazine (2-chloro-4-amino-6-isopropylamino-*s*-triazine), desisopropylatrazine (2-chloro-4-ethylamino-6-amino-*s*-triazine), and SIMAZINE (2-chloro-4,6-diethylamino-*s*-triazine).

Triazine degradation activity is encoded by a gene that is localized to a 21.5-kb *Eco*RI fragment, and more specifically to a 1.9-kb *Ava*I fragment, of the genome of *Pseudomonas* sp. ADP (ADP is strain designiation for Atrazine-degrading *Pseudomonas*) bacterium. Specifically, these genomic fragments encode proteins involved in *s*-triazine dechlorination. The rate of degradation of atrazine that results from the expression of these fragments in *E. coli* is comparable to that seen for native *Pseudomonas* sp. strain ADP; however, in contrast to what is seen with native *Pseudomonas* sp. strain ADP, this degradation in *E. coli* is unaffected by the presence of inorganic nitrogen sources like ammonium chloride. This is particularly advantageous for regions

contaminated with nitrogen-containing fertilizers or herbicides, for example. The expression of atrazine degradation activity in the presence of inorganic nitrogen compounds broadens the potential use of recombinant organisms for biodegradation of atrazine in soil and water.

Hydroxyatrazine formation in the environment was previously thought to result solely from the chemical hydrolysis of atrazine (Armstrong et al., Environ. Sci. Technol., 2, 683-689 (1968); deBruijn et al., Gene, 27, 131-149 (1984); and Nair et al., Environ. Sci. Technol., 26, 1627-1634 (1992)). Previous reports suggest that the first step in atrazine degradation by environmental bacteria is dealkylation. Dealkylation produces a product that retains the chloride moiety and is likely to retain its toxicity in the environment. In contrast to these reports, AtzA dechlorinates atrazine and produces a detoxified product in a one-step detoxification reaction that is amenable to exploitation in the remediation industry. There remains a need for atrazine-degrading enzymes with improved activity.

As used herein, the gene encoding a protein capable of dechlorinating atrazine and originally identified in *Pseudomonas* sp. strain ADP and expressed in *E. coli* is referred to as "*atz*A", whereas the protein that it encodes is referred to as "AtzA." Examples of the cloned wild type gene sequence and the amino acid sequence derived from the gene sequence are provided as SEQ ID NO:1 and SEQ ID NO:2 respectively. As also used herein, the terms atrazine chlorohydrolase (AtzA) protein, atrazine chlorohydrolase enzyme, or simply atrazine chlorohydrolase, are used interchangeably, and refer to an atrazine chlorohydrolase enzyme involved in the degradation of atrazine and similar molecules as discussed above.

A "homolog" of atrazine chlorohydrolase is an enzyme derived from the gene sequence encoding atrazine chlorohydrolase where the protein sequence encoded by the gene is modified by amino acid deletion, addition, substitution, or truncation but that nonetheless is capable of dechlorinating or deaminating *s*-triazine containing compounds. In addition, the homolog of atrazine chlorohydrolase (AtzA) has a nucleic acid sequence that is different

from the *atz*A sequence (SEQ ID NO:1) and produces a protein with modified biological properties or, as used herein, "altered enzymatic activities." These homologs include those with altered catalytic rates as quantitated by k_{cat} and K_m, altered substrate ranges, altered activities as compared to the native sequence in aqueous solutions, altered stability in solvents, altered active temperature ranges or altered reaction conditions such as salt concentration, pH, improved activity in a soil environment, and the like, as compared with the wild-type atrazine chlorohydrolase (AtzA) protein. Thus, provided that two molecules possess enzymatic activity to an *s*-triazine-containing substrate and one molecule has the gene sequence of *atz*A (SEQ ID NO:1), the other is considered a homolog of that sequence where 1) the gene sequence of the homolog differs from SEQ ID NO:1 such that there is at least one amino acid change in the protein encoded by SEQ ID NO:1 (i.e., SEQ ID NO:2); 2) the homolog has different enzymatic characteristics from the protein encoded by SEQ ID NO:1 such as, but not limited to, an altered substrate preference, altered rate of activity, or altered conditions for enzymatic activity such as temperature, pH, salt concentration or the like, as discussed *supra*; and 3) where the coding region of the nucleic acid sequence encoding the variant protein has at least 95% homology to SEQ ID NO:1.

As used herein, the terms "isolated and purified" refer to the isolation of a DNA molecule or protein from its natural cellular environment, and from association with other coding regions of the bacterial genome, so that it can be sequenced, replicated, and/or expressed. Preferably, the isolated and purified DNA molecules of the invention comprise a single coding region. Thus, the present DNA molecules are preferably those consisting of a DNA segment encoding a homolog of atrazine chlorohydrolase.

Using the nucleic acid encoding the wild-type *atzA* sequence and the amino acid sequence of the wild-type enzyme AtzA, similar atrazine degrading enzymes were identified in other bacteria. In fact, sequencing of the *atzA* gene in the other bacteria demonstrated a homology of at least 99% to the *atz*A sequence, suggesting little evolutionary drift (see SEQ ID NOS:12-16).

Homologs of the *atzA* gene could not be identified in the genomes of bacteria that did not metabolize atrazine. This information supports the theory that the *atzA* gene evolved to metabolize *s*-triazine-containing compounds.

The studies assessing the prevalence and homology of the *atz*A gene in a variety of bacterial genera also suggest that *atzA* is likely to be a relatively young, i.e. recently evolved gene. That the gene is recently evolved is supported by the attributes of the gene and the protein encoded by the gene. For example: (i) the gene has a limited *s*-triazine range that includes atrazine and the structurally analogous herbicide SIMAZINE, but does not act on all *s*-triazines; (ii) the gene has a high sequence homology to genes isolated from other bacteria that produce proteins with atrazine-degrading activity; (iii) is not organized with the *atzB* and *atzC* genes in a contiguous arrangement such as an operon; (iv) the gene lacks the type of coordinate genetic regulation seen, for example, in aromatic hydrocarbon biodegradative pathway genes; (v) the wild-type gene was isolated from a spill site containing high atrazine levels and (vi) it is suggested to have been environmentally undetectable until the last few years.

Genes involved in reactions common to most bacteria and mammals are more highly evolved and have attained catalytic proficiency closer to theoretical perfection. Genes that have evolved more recently have not had the evolutionary opportunity to maximize the level of catalytic efficiency that they could theoretically obtain. These enzymes are suboptimal. Suboptimal enzymes include enzymes that have a second order rate constant, k_{cat}/K_M, that is orders of magnitude below the diffusion-controlled limit of enzyme catalysis, 3×10^8 $M^{-1}s^{-1}$. These enzymes have the potential to evolve higher k_{cat}, lower K_m, or both. Enzymes with higher k_{cat}, lower K_m, or both would appear to have selective advantage as a biodegradative enzyme because less enzyme with higher activity would serve the same metabolic need and conserve ATP expended in enzyme biosynthesis. Optimized enzymes have the further advantage of having an improved commercial value resulting from their improved efficiency or improved activity under a defined set of conditions.

Thus, the *atzA* gene is, potentially, an *s*-triazine compound-degrading progenitor with the potential for improvement and modification. AtzA is a candidate for studies to generate homologs with improved activity, i.e., enhanced rate, altered pH preference, salt concentration and the like. The k_{cat}/K_M for atrazine chlorohydrolase purified from *Pseudomonas* ADP is 5×10^5 $M^{-1}s^{-1}$, 3 orders of magnitude below the theoretical catalytic limit. That all of the *atzA* homologous genes from a survey of atrazine-degrading bacteria are so structurally and catalytically similar suggest that the *atzA* gene and the AtzA protein can be improved and will be improved naturally over time. Indeed, most biodegradative enzymes are orders of magnitude below diffusion limiting enzyme rates and, under this hypothesis, are also candidates for gene and protein modifications.

In one embodiment of this invention, a method is disclosed for selecting or screening modified and improved *atzA* gene sequences that encode protein with improved enzymatic activity, whether the activity is enzymatic rate, using atrazine as a substrate, as compared to the wild-type sequence, or improved activity under any of a variety of reaction conditions including, but not limited to, elevated temperature, salt concentration, altered substrate range, solvent conditions, pH ranges, tolerance or stability to a variety of environmental conditions, or other reaction conditions that may be useful in bioremediation processes. The method preferably includes the steps of obtaining the wild-type *atz*A gene sequence, mutagenizing the gene sequence to obtain altered *atz*A sequences, selecting or screening for clones expressing altered AtzA activity and selecting gene sequences encoding AtzA protein with improved *s*-triazine-degrading activity.

As a first step for practicing the method of this invention, the wild-type *atz*A sequence (SEQ ID NO:1) is incorporated into a vector or into nucleic acid that is suitable for a particular mutagenesis procedure. The wild type *atz*A gene was first obtained as a 1.9-kb *Ava*I genomic fragment that encodes an enzyme that transforms atrazine to hydroxyatrazine, termed atrazine chlorohydrolase. Methods for obtaining this fragment are disclosed by de Souza

et al. (Appl. Environ. Microb. 61:3373-3378, (1995)). The gene, *atz*A, has one large ORF (open reading frame) and produces a translation product of about 473 amino acids. A particularly constant portion of this gene appears to occur at position 236 and end at position 1655 of SEQ ID NO:1. The wild-type *atz*A gene from *Pseudomonas* strain ADP includes 1419 nucleotides and encodes a polypeptide of 473 amino acids with an estimated M_r of 52,421 and a pI of 6.6. The gene also includes a typical *Pseudomonas* ribosome binding site, beginning with GGAGA, located 11 bp upstream from the proposed start codon. A potential stop codon is located at position 1655.

The wild-type *atz*A sequence can be obtained from a variety of sources including a DNA library, containing either genomic or plasmid DNA, obtained from bacteria believed to possess the *atz*A DNA. Alternatively the original isolate identified as containing the *atz*A DNA is described in U.S. Pat. No. 5,508,193 and can be accessed as a deposit from the American Type Culture Collection (ATCC No. 55464 Rockville, Maryland). Libraries can be screened using oligonucleotide probes, for example, to identify the DNA corresponding to SEQ ID NO:1. SEQ ID NO:1 can also be obtained by PCR (polymerase chain reaction) using primers selected using SEQ ID NO:1 and the nucleic acid obtained from the *atz*A-containing organism (ATCC No. 55464) deposited with the American Type Culture Collection.

Screening DNA libraries or amplifying regions from prokaryotic DNA using synthetic oligonucleotides is a preferred method to obtain the wild-type sequence of this invention. The oligonucleotides should be of sufficient length and sufficiently nondegenerate to minimize false positives. In a preferred strategy, the actual nucleotide sequence(s) of the probe(s) is designed based on regions of the *atz*A DNA, preferably outside of the reading frame of the gene (the translated reading frame begins at position 236 and ends at position 1655 of SEQ ID NO:1) that have the least codon redundancy.

Cloning of the open reading frame encoding *atz*A into the appropriate replicable vectors allows expression of the gene product, the AtzA enzyme, and makes the coding region available for further genetic engineering.

The types of mutagenesis procedures that are capable of generating a variety of gene sequences based on a parent sequence, *atz*A or a previously mutagenized or altered sequence of *atz*A, are known in the art and each method has a preferred vector format. In general, the mutagenesis procedures selected is one that generates at least one modified *atz*A sequence and preferably a population of modified *atz*A gene sequences. Selecting or screening procedures are used to identify preferred modified enzymes (i.e., homologs) from the pool of modified sequences.

There are a number of methods in use for creating mutant proteins in a library format from a parent sequence. These include the polymerase chain reaction (Leung, D.W. et al. Technique 1:11-15, (1989)), Bartel, D.P. et al. Science 261:1411-1418 (1993)), cassette mutagenesis (Arkin, A. et al. Proc. Natl. Acad. Sci. USA 89:7811-7815 (1992), Oliphant, A.R. et al., Gene 44:177-183 (1986), Hermes, J.D. et al., Proc. Natl. Acad. Sci. USA 87:696-700 (1990), Delgrave et al. Protein Engineering 6:327-331, (1993), Delgrave et al. Bio/Technology 11:1548-1552 (1993), and Goldman, ER et al., Bio/Technology 10:1557-1561 (1992)), as well as methods that exploit the standard polymerase chain reaction, including, but not limited to, DNA recombination during *in vitro* PCR (Meyerhans, A. et al., Nucl. Acids Res. 18:1687-1691 (1990), and Marton et al. Nucl. Acids Res. 19:2423-2426, 1991)), *in vivo* site specific recombination (Nissim et al. EMBO J. 13:692-698 (1994), Winter et al. Ann. Rev. Immunol. 12:433-55 (1994)), overlap extension and PCR (Hayashi et al. Biotechniques 17:310-315 (1994)), applied molecular evolution systems (Bock, L. C. et al., Nature 355:564-566 (1992), Scott, J. K. et al., Science 249: 386-390 (1990), Cwirla, S.E. et al. Proc. Natl. Acad. Sci. USA 87:6378-6382 (1990), McCafferty, J. et al. Nature 348:552-554 (1990)), DNA shuffling systems, including those reported by Stemmer et al. (Nature 370:389-391 (1994) and Proc. Natl. Acad. Sci. (USA) 91:10747-10751 (1994) and International Patent Application Publication Number WO 95/22625), and random *in vivo* recombination (see Caren et al. Bio/Technology 12: 433-55 (1994), Caloger et al. FEMS

Microbiology Lett. 97:41-44 (1992), International Patent Application Publication Numbers WO91/01087, to Galizzi and WO90/07576 to Radman, et al.).

Preferably, the method produces libraries with large numbers of mutant nucleic acid sequences that can be easily screened or selected without undue experimentation. Those skilled in the art will recognize that screening and/or selection methods are well documented in the art and those of ordinary skill in the art will be able to use the cited methods as well as other references similarly describing the afore-mentioned methods to produce pools of variant sequences. Preferred strategies include methods for screening for degradative activity of the *s*-triazine-containing compound on nutrient plates containing the homolog-encoding bacteria or by use of colormetric assays to detect the release of chlorine ions. Preferred selection assays include methods for selecting for homolog-containing bacterial growth on or in a *s*-triazine containing medium.

In a preferred method of this invention, gene shuffling, also termed recursive sequence recombination, is used to generate a pool of mutated sequences of the *atz*A gene. In this method the *atz*A gene, alone or in combination with the *atz*B gene, is amplified, such as by PCR, or, alternatively, multiple copies of the gene sequence (*atz*A and *atz*B) are isolated and purified. The gene sequence is cut into random fragments using enzymes known in the art, including DNAase I. The fragments are purified and the fragments are incubated with single or double-stranded oligonucleotides where the oligonucleotides comprise an area of identity and an area of heterology to the template gene or gene sequence. The resulting mixture is denatured and incubated with a polymerase to produce annealing of the single-stranded fragments at regions of identity between the single-stranded fragments. Strand elongation results in the formation of a mutagenized double-stranded polynucleotide. These steps are repeated at least once. In this gene shuffling technique, recombination occurs between substantially homologous, but non-identical, sequences of the *atz*A gene. In the studies provided in Example 2, the *atz*B gene was not gene-shuffled.

In the technique, published by Stemmer et al. (Nature, *supra*), the reassembled product is amplified by PCR and cloned into a vector. Clones containing the shuffled gene are next used in selection or screening assays. Example 2 discloses the use of a gene shuffling technique to generate pools of modified *atz*A sequences. The gene shuffling technique of Example 2 was modified based on the Stemmer et al. references. In this technique, an entire plasmid containing the *atz*A and *atz*B gene in a vector was treated with DNAase I and fragments between 500 and 2000 bp were gel purified. The fragments were assembled in a PCR reaction as provided in Example 2.

Once intact gene sequences are reassembled, they are incorporated into a vector suitable for expressing protein encoded by the reassembled nucleic acid, or as provided in Example 1, where the gene sequences are already in a vector, the vector can be incorporated directly into an organism suitable for replicating the vector. The vector containing the *atz*A gene is also preferably incorporated into a host suitable for expressing the *atz*A gene. The host, generally an *E. coli* species, is used in assays to screen or select for clones expressing the AtzA protein under defined conditions. The type of organism can be matched to the mutagenesis procedure and in Example 2, a preferred organism was the *E. coli* strain NM522.

The assays suitable for use in this invention can take any of a variety of forms for determining whether a particular protein produced by the organism containing the variant *atz*A sequences expresses an enzyme capable of dechlorinating or deaminating *s*-triazine compounds. Therefore, the types of assays that could be used in this invention include assays that monitor the degradation of *s*-triazine-containing compounds including ATRAZINE, SIMAZINE or MELAMINE using any of a variety of methods including, but not limited to, HPLC analysis to assess substrate degradation; monitoring clearing of precipitable *s*-triazine containing substrates, such as atrazine or TERBUTHYLAZINE, on solid media by bacteria containing the homologs of this invention; growth assays in media containing soluble substrate, monitoring the amount of chlorine released, as described by Bergman et al., Anal. Chem.,

29, 241-243 (1957) or the amount of nitrogen released; evaluating the derivitized product using gas chromatography and/or mass spectroscopy, solid agar plate assays with varied salt, pH substrate, solvent, or temperature conditions, colorimetric assays such as those provided by Epstein, J. ("Estimation of Microquantitation of Cyanide", (1947) *Analytical Chemistry* 19(4):272-276) and Habig and Jakoby ("Assays for Differentiation of Glutathione s-transferases, *Methods in Enzymology* 77:398-405) as well as radiolabelled assays to assess, for example, the release of radiolabel as a result of enzymatic activity.

In a preferred assay, clones are tested for their ability to degrade *s*-triazine-containing compounds such as atrazine, SIMAZINE, TERBUTHYLAZINE (2-chloro-4-(ethylamino)-6-(tertiary butyl-amino)-1,3,5-triazine), desethylatrazine, desisopropylatrazine, MELAMINE, and the like. In these assays, atrazine, or another insoluble *s*-triazine-containing substrate, is incorporated into a nutrient agar plate as the sole nitrogen source. Concentrations of atrazine or other *s*-triazine-containing compounds can vary in the plate from about 300 μg/ml to at least about 1000 μg/ml and in a preferred embodiment about 500 μg/ml atrazine is used on the plate. Many *s*-triazines are relatively insoluble compounds in water and a suspension in an agar plate produces a cloudy appearance. Bacteria capable of metabolizing the insoluble *s*-triazine-containing compounds produce a clearing on the cloudy agar plate. An exemplary assays is a modified assay disclosed by Mandelbaum et al. (Appl. Environ. Microbiol. 61:1451-1453, (1995)) and provided in Example 2. In these assays LB medium can be used with the atrazine because *E. coli* expressing AtzA homologs support atrazine-degrading activity in the presence of other nitrogen sources. The assay demonstrates atrazine degradation by observing clearing zones surrounding clones expressing homologs of AtzA.

Clones are selected from the insoluble substrate assay based on their ability to produce, for example, a clearing in the substrate-containing plates. Similarly, assay conditions can be modified such as, but not limited to, salt, pH, solvent, temperature, and the like, to select clones encoding AtzA homologs capable of degrading a substrate under a variety of test conditions. For example,

the pH of the assay can be altered to a pH range of about 5 to about 9. These assays would likely use isolated homolog protein to permit an accurate assessment of the effect of pH. The assay, or a modification of the assay, suitable for elevated temperatures (such as a soluble assay) can employ elevated temperature ranges, for example, between about 50° to about 80°C. The assays can also be modified to include altered salt concentrations including conditions equivalent to salt concentrations of about 2% to at least about 5% and preferably less than about 10% NaCl.

Clones identified as having altered enzymatic activity as compared with the native enzyme are further assessed to rule out if the apparent enhanced activity of the enzyme is the result of a faster or more efficient AtzA protein production or whether the effect observed is the result of an altered *atz*A gene sequence. For example, in Example 2, the *atzA* was expressed to a high level using pUC18 as a preferred method to rule out higher *in vivo* activity due to increased expression.

Once triazine-degrading colonies are identified with the desired characteristics, the AtzA homologs are isolated for further analysis. Clones containing putative faster enzyme(s) can be picked, grown in liquid culture, and the protein homolog can be purified, for example, as described (de Souza et al. J. Bacteriology, 178:4894-4900 (1996)). The genes encoding the homologs can be modified, as known in the art, for extracellular expression or the homologs can be purified from bacteria. An exemplary method for protein purification is provided in Example 4. In a preferred method, protein was collected from bacteria using ammonium sulfate precipitation and further purified by HPLC (see for example, de Souza et al., App. Envir. Microbio. 61:3373-3378 (1995)).

Using these methods, a number of homologs were identified. Homologs can be identified using the assays discussed in association with this invention including the precipitable substrate assays on solid agar as described by Mandelbaum, et al. (*supra*). Homologs identified using the methods of Example 2 were separately screened for atrazine-degrading activity, for enhanced TERBUTHYLAZINE-degrading activity and for activity against other

s-triazine-containing compounds. An assay for TERBUTHYLAZINE degrading activity is provided in Example 6. Two homologs were found to have at least a 10 fold higher activity and contained 8 different amino acids than the native AtzA protein (A7 and T7, see Figs. 1-4). A subsequent round of DNA shuffling starting with the homolog gene sequence yielded further improvements in activity (A11 and A13 corresponding to nucleic acid SEQ ID NOS: 7-10 and SEQ ID NO:11 respectively). This enzyme and other AtzA homologs (clones A40, A42, A44, A46, A60 corresponding to nucleic acid SEQ ID NOS: 17-21 and to protein SEQ ID NOS: 22-26, respectively) represent catabolic enzymes modified in their biological activity. Preferred homologs identified in initial studies include A7, T7, A11, A44, and A46.

Homologs were also identified with altered substrate activity. Both homologs T7 and A7 were able to degrade TERBUTHYLAZINE better than the wild-type enzyme. Other homologs capable of degrading TERBUTHYLAZINE include A42, A44, A46 and A60.

Atrazine chlorohydrolase converts a herbicide to a non-toxic, non-herbicidal, more highly biodegradable compound and the kinetic improvement of the homologs has important implications for enzymatic environmental remediation of this widely used herbicide. Less protein is required to dechlorinate the same amount of atrazine. Importantly, the protein can also be used for degradation of the *s*-triazine-compound TERBUTHYLAZINE.

This invention also relates to nucleic acid and protein sequences identified from the homologs of this invention. Peptide and nucleic acid fragments of these sequences are also contemplated and those skilled in the art can readily prepare peptide fragments, oligonucleotides, probes and other nucleic acid fragments based on the sequences of this invention. The homologs of this invention include those with an activity different from the native atrazine chlorohydrolase (AtzA) protein. As noted *supra*, an activity that is different from the native atrazine chlorohydrolase protein includes enzymatic activity that is improved or is capable of functioning under different conditions such as salt

concentration, temperature, altered substrate, or the like. Preferably, the DNA encoding the homologs hybridize to a DNA molecule complementary to the wild-type coding region of a DNA molecule encoding wild-type AtzA protein, such as the sequence provided in SEQ ID NO:1, under high to moderate stringency hybridization conditions. The homologs preferably have a homology of at least 95% to SEQ ID NO:1. As used herein, "high stringency hybridization conditions" refers to, for example, hybridization conditions in buffer containing 0.25 M Na_2HPO_4 (pH 7.4), 7% sodium dodecyl sulfate (SDS), 1% bovine serum albumin (BSA), 1.0 mM ethylene diamine tetraacetic acid (EDTA, pH 8) at 65°C, followed by washing 3x with 0.1% SDS and 0.1x SSC (0.1x SSC contains 0.015 M sodium chloride and 0.0015 M trisodium citrate, pH 7.0) at 65°C.

A number of homologs have been identified using the methods of this invention. For example, SEQ ID NO:3 is the gene sequence of a homolog A7 of the *atz*A gene that shows enhanced atrazine degradation activity and, surprisingly, also demonstrated enhanced TERBUTHYLAZINE degradation activity. TERBUTHYLAZINE degradation experiments are provided in Example 6. The amino acid sequence of the enzyme encoded by SEQ ID NO:3 identified as SEQ ID NO:5. SEQ ID NO: 4 is the gene sequence of the homolog T7 of the *atz*A gene that shows enhanced atrazine degradation activity and enhanced TERBUTHYLAZINE degradation activity. A summary of the TERBUTHYLAZINE degradation activity for T7 and A7 is provided in Example.6. SEQ ID NO:6 provides the amino acid sequence of the homolog encoded by SEQ ID NO:4. Fig. 1 provides the nucleotide sequence alignment of wild type *atz*A from SEQ ID NO:1 with SEQ ID NO:3 and Fig.2 provides the nucleotide sequence alignment of SEQ ID NO:1 with SEQ ID NO:4. Fig. 3 provides the amino acid sequence alignment of SEQ ID NO:2, the amino acid sequence of the protein encoded by SEQ ID NO:1, with SEQ ID NO:5 and Fig. 4 provides the amino acid sequence alignment of SEQ ID NO:2 with SEQ ID NO:6. A review of the sequences encoding A7 and T7 indicate that both homologs have a total of 8 amino acid changes relative to native AtzA (SEQ ID NO:2). Seven amino acid changes are common to both A7 and T7. The nucleic

acid sequences of other homologs with altered activity include A40 (nucleic acid SEQ ID NO:17; amino acid sequence SEQ ID NO:22); A42 (nucleic acid SEQ ID NO:18; amino acid sequence SEQ ID NO:23); A44 (nucleic acid SEQ ID NO:19; amino acid sequence SEQ ID NO:24); A46 (nucleic acid SEQ ID NO:20; amino acid sequence SEQ ID NO:25); and A60 (nucleic acid SEQ ID NO:21; amino acid sequence SEQ ID NO:26).

Without intending to limit the scope of this invention, the success attributed to the identification of homologs of AtzA may be based on the recognition that this protein is not evolutionarily mature. Therefore, not all gene sequences are good candidates as the starting material for identifying a number of biological variants of a particular protein and similarly, not all enzymes are amenable to the order of magnitude of rate enhancement by directed evolution using DNA shuffling or other methods. Without intending to limit the scope of this invention, it is believed that some enzymes are already processing substrates at their theoretical rate limit. In these cases, catalysis is limited by the physical diffusion of the substrate onto the catalytic surface of the enzyme. Thus, changes in the enzyme would not likely improve the rate of catalysis. Examples of enzymes that operate at or near catalytic "perfection" are triosephosphate isomerase, fumarase, and crotonase (available from the GenBank database system). Even biodegradative enzymes that hydrolyze toxic substrates fall into this class. For example, the phosphotriesterase that hydrolyzes paraoxon operates near enough to the diffusion limit and suggests that it would not be a good candidate for mutagenic methods to improve the catalytic rate constant of the enzyme with its substrate (see Caldwell et al., Biochem. 30:7438-7444 (1991)).

The gene sequences of this invention can be incorporated into a variety of vectors. Preferably, the vector includes a region encoding a homolog of AtzA and the vector can also include other DNA segments operably linked to the coding sequence in an expression cassette, as required for expression of the homologs, such as a promoter region operably linked to the 5' end of the coding DNA sequence, a selectable marker gene, a reporter gene, and the like.

The present invention also provides recombinant cells expressing the homologs of this invention. For example, DNA that expresses the homologs of this invention can be expressed in a variety of bacterial strains including *E. coli* sp. strains and *Pseudomonas* sp. strains. Other organisms include, but are not limited to, *Rhizobium, Bacillus, Bradyrhizobium, Arthrobacter, Alcaligenes,* and other rhizosphere and nonrhizosphere soil microbe strains.

In addition to prokaryotes, eukaryotic microbes such as filamentous fungi or yeast are suitable hosts for vectors encoding *atz*A or its homologs. *Saccharomyces cerevisiae*, or common baker's yeast, is the most commonly used among lower eukaryotic host microorganisms. However, a number of other genera, species, and strains are commonly available and useful herein, such as *Schizosaccaromyces pombe, Kluyveromyces* hosts such as, *e.g.*, *K. lactis, K. fragilis, K. bulgaricus, K. thermotolerans,* and *K. marxianus, Pichia pastoris, Candida, Trichoderma reesia, Neurospora crassa*, and filamentous fungi such as, e.g., *Neurospora, Penicillium, Tolypocladium*, and *Aspergillus* hosts such as *A. nidulans.*

Prokaryotic cells used to produce the homologs of this invention are cultured in suitable media, as described generally in Maniatis et al., Molecular Cloning: A Laboratory Manual; Cold Spring Harbor Press: Cold Spring Harbor, NY (1989). Any necessary supplements may also be included at appropriate concentrations that would be known to those skilled in the art. In general the *E. coli* expressing the homologs of this invention are readily cultured in LB media (see Maniatis, *supra*). The culture conditions, such as temperature, pH, and the like, are those previously used with the host cell selected for expression, and will be apparent to those skilled in the art. Induction of cells to express the AtzA protein is accomplished using the procedures required by the particular expression system selected. The host cells referred to in this disclosure are generally cultured *in vitro*. Cells are harvested, and cell extracts are prepared, using standard laboratory protocols.

This invention also relates to isolated proteins that are the product of the gene sequences of this invention. The isolated proteins are protein

homologs of the wild-type atrazine chlorohydrolase enzyme despite their potential for altered substrate preference. The protein can be isolated in a variety of methods disclosed in the art and a preferred method for isolating the protein is provided in Examples 4 and 5 and in the publications of de Souza et al. (*supra*).

The wild-type AtzA protein acts on Atrazine, desethylatrazine, Desisopropylatrazine and SIMAZINE but did not degrade Desethyldesisopropylatrazine or MELAMINE and only poorly degraded TERBUTHYLAZINE. Homologs identified in this invention have a spectrum of substrate preferences identical to the wild-type AtzA protein and in addition, for example, are able to degrade other substrates such as TERBUTHYLAZINE. That homologs were identified that were capable of degrading two different *s*-triazine-containing compounds suggests that the methods of this invention can be used on the wild-type progenitor *atz*A gene or on the homologs produced by this invention to produce even more useful proteins for environmental remediation of *s*-triazine-containing compounds. Example 7 provides an assay for detecting degradation, including deamination, of a soluble *s*-triazine-containing compound.

Various environmental remediation techniques are known that utilize high levels of proteins. Bacteria or other hosts expressing the homologs of this invention can be added to a remediation mix or mixture in need of remediation to promote contaminate degradation. Alternatively, isolated AtzA homologs can be added. Proteins can be bound to immobilization supports, such as beads, particles, films, etc., made from latex, polymers, alginate, polyurethane, plastic, glass, polystyrene, and other natural and man-made support materials. Such immobilized protein can be used in packed-bed columns for treating water effluents. The protein can be used to remediate liquid samples, such as contaminated water, or solids. The advantage of some of the homologs identified thus far indicate that the homologs demonstrate an ability to degrade more than one substrate and to degrade the substrate at a faster rate or under different reaction conditions from the native enzyme.

All references and publications cited herein are expressly incorporated by reference into this disclosure. The invention will be further

described by reference to the following detailed examples. Particular embodiments of this invention will be discussed in detail and reference has been made to possible variations within the scope of this invention. There are a variety of alternative techniques and procedures available to those of skill in the art which would similarly permit one to successfully perform the intended invention that do not detract from the spirit and scope of this invention.

Example 1
Isolation of Wild-type atzA gene from *Pseudomonas* sp. strain ADP

Bacterial strains and growth conditions.

Pseudomonas sp. strain ADP (Mandelbaum et al., Appl. Environ. Microbiol., 59, 1695-1701 (1993)) was grown at 37°C on modified minimal salt buffer medium, containing 0.5% (wt/vol) sodium citrate dihydrate. The atrazine stock solution was prepared as described in Mandelbaum et al., Appl. Environ. Microbiol., 61, 1451-1457 (1995)). *Escherichia coli* DH5α was grown in Luria-Bertani (LB) or M63 minimal medium, which are described in Maniatis et al., Molecular Cloning: A Laboratory Manual; Cold Spring Harbor Press: Cold Spring Harbor, NY (1989). Tetracycline (15 μg/ml), kanamycin (20 μg/ml), and chloramphenicol (30 μg/ml) were added as required.

To construct the *Pseudomonas* sp. strain ADP genomic library, total genomic DNA was partially digested with *Eco*RI, ligated to the *Eco*RI-digested cosmid vector pLAFR3 DNA, and packaged *in vitro*. The completed genomic DNA library contained 2000 colonies.

To identify the atrazine degrading clones, the entire gene library was replica-plated onto LB medium containing 500 μg/ml atrazine and 15 μg/ml tetracycline. Fourteen colonies having clearing zones were identified. All fourteen clones degraded atrazine, as determined by HPLC analysis. Cosmid DNA isolated from the fourteen colonies contained cloned DNA fragments which were approximately 22 kb in length. The fourteen clones could be subdivided into six groups on the basis of restriction enzyme digestion analysis using *Eco*RI. All fourteen clones, however, contained the same 8.7 kb *Eco*RI

fragment. Thirteen of the colonies, in addition to degrading atrazine, also produced an opaque material that surrounded colonies growing on agar medium. Subsequent experiments indicated that the opaque material only was observed in *E. coli* clones which accumulated hydroxyatrazine. Thus, the cloudy material surrounding *E. coli* pMD2-pMD4 colonies was due to the deposition of hydroxyatrazine in the growth medium. The one colony that degraded atrazine without the deposition of the opaque material was selected for further analysis. The clone from this colony was designated pMD1.

Example 2
Mutagenesis Procedure

Gene Shuffling. Atz A and B genes were subcloned from pMD1 into pUC18. The two inserts were reduced in size to remove extraneous DNA. A 1.9 kb AvaI fragment containing *atzA* was end-filled and cloned into the end-filled AvaI site of pUC18. A 3.9 kb ClaI fragment containing *atzB* was end-filled and cloned into the HincII site of pUC18. The gene *atzA* was then excised from pUC18 with EcoRI and BamHI, *AtzB* with BamHI and HindIII, and the two inserts were co-ligated into pUC18 digested with EcoRI and HindIII. The result was a 5.8 kb insert containing *AtzA* and *AtzB* in pUC18 (total plasmid size 8.4 kb).

Recursive sequence recombination was performed by modifications of existing procedures (Stemmer, W., Proc. Natl. Acad. Sci. USA 91:10747-10751 (1994) and Stemmer, W. Nature 370:389-391 (1994)). **[Mervyn, do you know more now about what was done?]** The entire 8.4 kb plasmid was treated with DNAase I in 50 mM Tris-Cl pH 7.5, 10 mM $MnCl_2$ and fragments between 500 and 2000 bp were gel purified. The fragments were assembled in a PCR reaction using Tth-XL enzyme and buffer from Perkin Elmer, 2.5 mM MgOAc, 400 μM dNTPs and serial dilutions of DNA fragments. The assembly reaction was performed in an MJ Research "DNA Engine" thermocycler programmed with the following cycles:

1	94°C, 20 seconds
2	94°C, 15 seconds
3	40°C, 30 seconds
4	72°C, 30 seconds + 2 seconds per cycle
5	go to step 2 39 more times
6	4°C

The *atz*A gene could not be amplified from the assembly reaction using the polymerase chain reaction, so instead DNA from the reaction was purified by standard phenol extraction and ethanol precipitation methods and digested with KpnI to linearize the plasmid (the KpnI site in pUC18 was lost during subcloning, leaving only the KpnI site in *atzA*). Linearized plasmid was gel-purified, self-ligated overnight and transformed into *E coli* strain NM522.

Serial dilutions of the transformation reaction were plated onto LB plates containing 50 μg/ml ampicillin, the remainder of the transformation was stored in 25% glycerol and frozen at -80°C. Once the transformed cells were titered, the frozen cells were plated at a density of between 200 and 500 on 150 mm diameter plates containing 500 μg/ml atrazine or another substrate and grown at 37°C.

Atrazine at 500 μg/ml forms an insoluble precipitate creating a cloudy appearance on the agar plate. The solubility of atrazine is about 30 μg/ml, therefore for precipitable substrate assays, such as the assay disclosed here, the atrazine concentration should be preferably greater than 30 μg/ml. Atrazine or hydroxyatrazine were incorporated in solid LB or minimal medium, as described in Mandelbaum et al., Appl. Environ. Microbiol., 61, 1451-1457 (1995), at a final concentration of 500 μg/ml to produce an opaque suspension of small particles in the clear agar. AtzA and the homologs with atrazine-degrading activity convert atrazine into a soluble product. The degradation of atrazine or hydroxyatrazine by wild-type and recombinant bacteria was indicated by a zone of clearing surrounding colonies. The more active the homolog, the more rapidly a clear halo formed on atrazine-containing plates. Positive colonies that most rapidly formed the largest clear zones were selected initially for further analysis. The (approximately) 40 best colonies were picked, pooled, grown in the presence of 50 μg/ml ampicillin and plasmid prepared from them. More

efficient enzymes can also be tested using atrazine concentrations greater than 500 μg/ml.

The entire process (from DNAase-treatment to plating on atrazine plates) was repeated 4 times as a method for further improving on the rate of enzymatic activity. In several experiments, cells were plated on plates containing 500 μg/ml atrazine and on plates containing 500 μg/ml of the atrazine analogue TERBUTHYLAZINE.

Other compounds can be tested in similar assays replacing atrazine (2-chloro-4-ethlyamino-6-isopropylamino-1,3,5-*s*-triazine) for the following compounds: desethylatrazine (2-chloro-4-amino-6-isopropylamino-*s*-triazine), deisopropylatrazine (2-chloro-4-ethylamino-6-amino-*s*-triazine), hydroxyatrazine (2-hydroxy-4-ethylamino-6-isopropylamino-*s*-triazine), desethylhydroxyatrazine (2-hydroxy-4-amino-6-isopropylamino-*s*-triazine), desisopropylhydroxyatrazine (2-hydroxy-4-amino-6-isopropylamino-*s*-triazine), desethyldesisopropylatrazine (2-chloro-4,6-diamino-*s*-triazine), SIMAZINE (2-chloro-4,6-diethylamino-*s*-triazine), TERBUTHYLAZINE (2-chloro-4-ethylamino-6-terbutylamino-*s*-triazine, and MELAMINE (2,4,6-triamino-*s*-triazine) were obtained from Ciba Geigy Corp., Greensboro, N.C. Ammelide (2,4-dihydroxy-6-amino-*s*-triazine), ammeline (2-hydroxy-4,6,-diamino-*s*-triazine) were obtained from Aldrich Chemical Co., Milwaukee, WI.

Example 3
DNA Sequencing of Wild-Type atzA and Homolog atzA genes

DNA Sequencing. The nucleotide sequence of the approximately 1.9-kb *Ava*I DNA fragment in vector pACYC184, designated pMD4, or the homologs in pUC18 or another vector was determined using both DNA strands. DNA was sequenced by using a PRISM Ready Reaction DyeDeoxy Terminator Cycle Sequencing kit (Perkin-Elmer Corp., Norwalk, CT) and a ABI Model 373A DNA Sequencer (Applied Biosystems, Foster City, CA). Nucleotide sequence was determined initially by subcloning and subsequently by using primers designed based on sequence information obtained from subcloned DNA fragments. The GCG sequence analysis software package (Genetics Computer

Group, Inc., Madison, WI) was used for all DNA and protein sequence comparisons. Radiolabelled chemicals were obtained from Ciba Geigy Corp., Greensboro, N.C.

Example 4
Protein Purification of AtzA or Homologs

E. coli transformed with a vector containing the wild type *atz*A gene or alternatively with a homolog, in a vector capable of directing expression of the gene as a protein, was grown overnight at 37°C in eight liters of LB medium containing 25 µg/ml chloramphenicol. The culture medium was centrifuged at 10,000 x g for 10 minutes at 4°C, washed in 0.85% NaCl, and the cell pellet was resuspended in 50 ml of 25 mM MOPS buffer (3-[N-morpholino]propane-sulfonic acid, pH 6.9), containing phenylmethylsulfonylfluoride (100 µg/ml). The cells were broken by three passages through an Amicon French Pressure Cell at 20,000 pounds per square inch (psi) at 4°C. Cell-free extract was obtained by centrifugation at 10,000 x g for 15 minutes. The supernatant was clarified by centrifugation at 18,000 x g for 60 minutes and solid NH_4SO_4 was added, with stirring, to a final concentration of 20% (wt/vol) at 4°C. The solution was stirred for 30 minutes at 4°C and centrifuged at 12,000 x g for 20 minutes. The precipitated material was resuspended in 50 ml of 25 mM MOPS buffer (pH 6.9), and dialyzed overnight at 4°C against 1 liter of 25 mM MOPS buffer (pH 6.9).

Where purified protein was desired, the solution was loaded onto a Mono Q HR 16/10 Column (Pharmacia LKB Biotechnology, Uppsala, Sweden). The column was washed with 25 mM MOPS buffer (pH 6.9), and the protein was eluted with a 0-0.5 M KCl gradient. Protein eluting from the column was monitored at 280 nm by using a Pharmacia U.V. protein detector. Pooled fractions containing the major peak were dialyzed overnight against 1 liter 25 mM MOPS buffer (pH 6.9). The dialyzed material was assayed for atrazine degradation ability by using HPLC analysis (see above) and analyzed for purity by sodium dodecyl sulfate (SDS) polyacrylamide gel electrophoreses (Laemlli).

Protein Verification: Protein subunit sizes were determined by SDS polyacrylamide gel electrophoresis by comparison to known standard proteins, using a Mini-Protean II gel apparatus (Biorad, Hercules, CA). The size of the holoenzyme was determined by gel filtration chromatography on a Superose 6 HR (1.0 x 30.0 cm) column, using an FPLC System (Pharmacia, Uppsala, Sweden). The protein was eluted with 25 mM MOPS buffer (pH 6.9) containing 0.1 M NaCl. Proteins with known molecular weights were used as chromatography standards. Isoelectric point determinations were done using a Pharmacia Phast-Gel System and Pharmacia IEF 3-9 media. A Pharmacia broad-range pI calibration kit was used for standards.

Enzyme Kinetics. Purified AtzA protein and homologs of the protein at 50 μg/ml, were separately added to 500 μl of different concentrations of atrazine (23.3 μM, 43.0 μM, 93 μM, 233 μM, and 435 μM in 25 mM MOPS buffer, pH 6.9) or another *s*-triazine-containing compound and reactions were allowed to proceed at room temperature for 2, 5, 7, and 10 minutes. The reactions were stopped by boiling the reaction tubes at specific times, the addition of 500 μl acetonitrile and rapid freezing at -80°C. Thawed samples were centrifuged at 14,000 rpm for 10 minutes, the supernatants were filtered through a 0.2 μM filter, and placed into crimp-seal HPLC vials. HPLC analysis was done as described above. Based on HPLC data, initial rates of atrazine degradation and hydroxyatrazine formation were calculated and Michaelis Menton and Lineweaver Burke plots were constructed.

Effect of simple nitrogen sources on atrazine degradation. From experiments done with *Pseudomonas* species strain ADP on solid media with 500 ppm atrazine and varying concentrations of ammonium chloride, ammonium chloride concentrations as low as 0.6-1.2 mM were sufficient to inhibit visible clearing on the plates, even after 2 weeks of incubation either at 28°C or 37°C. With similar experiments using *E. coli* DH5α (pMD1 or pMD2) and other *E. coli* strains, atrazine degradation was observed in the presence of ammonium chloride concentrations as high as 48 mM. This value is almost 40-80 fold higher than the wild-type tolerance for ammonium chloride with

concomitant atrazine degradation. Therefore, it was not necessary to use media free of exogenous ammonia in the screening assays.

Example 5
Further characterization of the enzymatic activity of the homologs

Analysis of atrazine metabolism by *E. coli* clones. The extent and rate of atrazine degradation was determined in liquid culture. *E. coli* clones containing plasmids capable of expressing the homologs were compared to *Pseudomonas* sp. strain ADP for their ability to transform ring-labelled [^{14}C]-atrazine to water-soluble metabolites. This method, which measures [^{14}C]-label partitioning between organic and aqueous phases, had previously been used with *Pseudomonas* sp. ADP to show the transformation of atrazine to metabolites that partition into the aqueous phase, in Mandelbaum et al., Appl. Environ. Microbiol., 61, 1451-1457 (1995). When *Pseudomonas* sp. strain ADP or *E. coli* capable of expressing the homologs of this invention were incubated for 2 hours with [^{14}C]-atrazine, 98%, 97%, 88%, and 92%, respectively, of the total recoverable radioactivity was found in the aqueous phase. Greater than 90% of the initial radioactivity was accounted for as atrazine plus water soluble metabolites, indicating that little or no $^{14}CO_2$ was formed. In contrast, forty-four percent of the radioactivity was lost from the *Pseudomonas* ADP culture after 18.5 hours. In previous studies done with *Pseudomonas* sp. strain ADP and ring-labelled ^{14}C-atrazine, radiolabel was lost from culture filtrates as $^{14}CO_2$ (see, e.g., Mandelbaum et al., Appl. Environ. Microbiol., 61, 1451-1457 (1995)). Retention of the radiolabel is indicative of lack or inhibition of enzymatic activity. While these studies were performed for AtzA, similar studies are used to assess the activity of the homologs of this invention.

Example 6
Assays to detect homologs of AtzA on TERBUTHYLAZINE

TERBUTHYLAZINE was incorporated in solid LB medium at a final concentration of about 400-500 µg/ml to produce an opaque suspension of sample particles in the clear agar. The degradation of terbuthyalazine by

recombinant bacteria was indicated by a zone of clearing surrounding the colonies. HPLC analysis was performed with a Hewlett Packard HP 1090 Liquid Chromatograph system equipped with a photodiode array detector and interfaced to an HP 79994A Chemstation. TERBUTHYLAZINE and its metabolites were resolved by using an analytical C^{18} reverse-phase Nova-Pak HPLC column (4-μm-diameter spherical packing, 150 by 3.9 mm; Waters Chromatography, Milford, Mass.) and an acetonitrile (ACN) gradient, in water, at a flow rate of 1.0 ml min^{-1}. Linear gradients of 0 to 6 min, 10 to 25% ACN; 6 to 21 min, 25 to 65% ACN; 21 to 23 min, 65 to 100% ACN; and 23 to 25 min, 100% ACN were used. Spectral data of the column eluent were acquired between 200 and 400 nm (12-nm bandwidth per channel) at a sampling frequency of 640 ms. Spectra were referenced against a signal of 500 nm.

Comparative results of an assay to assess TERBUTHYLAZINE degradation is provided in Figures 7 and 8. Figure 7 (a) provides a histogram demonstrating the relative percentage of TERBUTHYLAZINE remaining in samples tested while Figure 7(b) provides a measure of the production of hydroxyterbuthylazine as a measure of TERBUTHYLAZINE degradation. Sample 1 is a control sample without enzyme. Sample 2 uses a two fold excess of AtzA protein as compared to the concentration of homolog added in Sample 3 and Sample 4. Sample 3 employed the T7 homolog (SEQ ID NO:6) and Sample 4 employed the A7 homolog (SEQ ID NO:5). Results were determined by HPLC as described above. Figure 8(a) provides the percentage of TERBUTHYLAZINE remaining after a 15 minute exposure to homologs A7, A11, and T7. Samples 1-10 refer to the effect of homolog activity in the presence of 50 uM of: Manganese (1); Mangnesium (2), EDTA (3); cobalt (4); zinc (5); iron (6); copper (7); nickel (8); no metal (9); or no eznyme (10). Figure 8(b) provides the relative amount of hydroxyterbuthylazine as a measure of TERBUTHYLAZINE degradation for homologs A7 (solid bar), A11 (hatched bar), or T7 (open bar) in the presence or absence of additives 1-10 (*supra*).

Example 7
Assays to detect homologs of AtzA on "MELAMINE"

"MELAMINE" (2, 4, 6-triamino -*s*-triazine) at a concentration of at least about 1 mM to about 5 mM and preferably about 2 mM MELAMINE is incorporated into solid minimal nutrient media as the sole nitrogen source. Bacteria are distributed on the plate and growth of the organisms is indicative of their ability to degrade MELAMINE, thereby releasing ammonia for growth. Growth is evidence of the ability of the organisms expressing the homologs of this invention to deaminate MELAMINE. There is more than one nitrogen-containing group in MELAMINE. Therefore the selection of larger colonies on MELAMINE containing solid minimal nutrient media could be used to select for faster MELAMINE-degrading homologs.

A comparison of the nucleic acid sequence from a wild type MELAMINE degrading *Pseudomonas* NRRLB 12227 strain as compared to the *atz*A gene sequence indicated a homology of more than 90% over a 500 base pair sequence obtained from NRRLB using primer selected that were internal to atzA suggesting that homologs of *atz*A could be identified that degrade "MELAMINE." This strain did not degrade atrazine. Moreover, homologs identified using the methods of Example 2 are subjected to further mutagenesis and colonies capable of growing in MELAMINE can be identified. Colonies containing the protein AtzA are tested for growth in MELAMINE under identical conditions. Other *s*-triazine containing compounds such as the pesticides available under the tradenames "AMETRYN", "PROMETRYN", "PROMETRON", "ATRATON" and "CYROMAZINE" could also function as substrates for other homologs of this invention.

It will be appreciated by those skilled in the art that while the invention has been described above in connection with particular embodiments and examples, the invention is not necessarily so limited and that numerous other embodiments, examples, uses, modifications and departures from the

embodiments, examples and uses may be made without departing from the inventive scope of this application.